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Details of Mannitol Transport in *Escherichia coli* Elucidated by Site-Specific Mutagenesis and Complementation of Phosphorylation Site Mutants of the Phosphoenolpyruvate-Dependent Mannitol-Specific Phosphotransferase System†

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ABSTRACT: The mannitol transport protein (EII^{mtl}) carries out translocation with concomitant phosphorylation of mannitol from the periplasm to the cytoplasm, at the expense of phosphoenolpyruvate (PEP). The phosphoryl group which is needed for this group translocation is sequentially transferred from PEP via two phosphorylation sites, located exclusively on the C-terminal cytoplasmic domain, to mannitol. Oligonucleotide-directed mutagenesis was used to investigate the precise role of these sites in phosphoryl group transfer, by producing specific amino acid substitutions. The first phosphorylation site, His-554 (P1), was replaced by Ala, which renders the EII-H554A completely inactive in PEP-dependent mannitol phosphorylation, but not in mannitol/mannitol 1-phosphate exchange. The P2 site mutant, EII-C384S, was inactive both in the mannitol phosphorylation reaction and in the exchange reaction, due to replacement of the essential Cys-384 by Ser. Although EII-H554A and EII-C384S were both catalytically inactive in the PEP-dependent phosphorylation, EII-C384S was able to restore up to 55% of the wild-type mannitol phosphorylation activity with the EII-H554A mutant, indicating a direct phosphotransfer between two subunits. These phosphorylation data together with the data obtained from mannitol/mannitol phosphate exchange kinetics, after mixing EII-H554A and EII-C384S, indicated the formation of functionally stable heterodimers, which consist of an EII-H554A and an EII-C384S monomer.

The bacterial phosphotransferase system (PTS)[1] mediates transport with concomitant phosphorylation of hexoses and hexitols. It consists of two general phosphocarriers, EI and HPr, and several sugar-specific transport proteins, the enzyme II or enzyme II/III pairs, which become phosphorylated during sugar uptake (Postma & Lengeler, 1985; Robillard & Lolkema, 1988). The mannitol-specific transport protein (EII^{mtl}), an integral membrane protein, carries out mannitol translocation and phosphorylation from the outside to the inside of the cell at the expense of phosphoenolpyruvate (PEP) as illustrated in Scheme I.

Scheme I

$$PEP + HPr \overset{EI}{\leftrightarrow} P\text{-}HPr + pyruvate \quad (1)$$

$$P\text{-}HPr + EII^{mtl} \leftrightarrow P\text{-}His554\text{-}EII^{mtl} + HPr \quad (2)$$

$$P\text{-}His554\text{-}EII^{mtl} \leftrightarrow P\text{-}Cys384\text{-}EII^{mtl} \quad (3)$$

$$P\text{-}Cys384\text{-}EII^{mtl} + mtl_{out} \leftrightarrow EII^{mtl} + mtl\text{-}P_{in} \quad (4)$$

A mannitol/mannitol 1-phosphate exchange reaction (Scheme II) has also been shown to be catalyzed by EII^{mtl}.

Scheme II

$$mtl\text{-}P + {}^{*}mtl \overset{EII^{mtl}}{\leftrightarrow} {}^{*}mtl\text{-}P + mtl$$

EII^{mtl} was the first of this class of transport proteins to be purified and to have its gene *mtl*A cloned and sequenced (Jacobson et al., 1979; Lee & Saier, 1983). It is a single polypeptide of 67 893 daltons which consists of two separate domains: the N-terminal domain is embedded in the membrane and contains the binding site for mannitol; the C-terminal domain is exposed in the cytoplasm and binds phospho-HPr and passes on the phosphoryl group to the sugar.

The absence of a separate mannitol-specific EIII in the mannitol PTS of *Escherichia coli* and the presence of a larger cytoplasmic domain, compared to various EII/III pairs, led to the proposal that *E. coli* EII^{mtl} should contain an EIII-like domain. This implies that this single EII species should also possess two phosphorylation sites, one of which should be on the covalently linked EIII-like domain. Both *E. coli* EII^{mtl} phosphorylation sites have been identified by labeling with [^{32}P]phosphoenolpyruvate followed by digestion with proteolytic enzymes and HPLC purification of the labeled peptides. Sequence analysis showed that both peptides were derived from the C-terminal domain of EII^{mtl} (Pas & Robillard, 1988). The first phosphorylation site (P1) was located in peptide (Leu-541 to Lys-560) containing His-554. A homologous peptide was found recently in $EIII^{mtl}$ of the mannitol PTS in *Staphylococcus aureus* and *Staphylococcus carnosus* (Reiche et al., 1988; Fischer et al., 1989). These data, together with the results of the construction and characterization of *E. coli* EII^{mtl} deletion mutants reported by Grisafi et al. (1989), the complementation experiments between a truncated EII^{mtl} and a partially purified C-terminal domain (Stephan et al., 1989), or an expressed large C-terminal fragment (White & Jacobson,

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[1] Abbreviations: PTS, phosphotransferase system; PEP, phosphoenolpyruvate; EI, enzyme I; $EII^{mtl,nag,glc,bgl}$, mannitol-, *N*-acetylglucosamine-, glucose-, and β-glucoside-specific enzyme II's of the PTS; $EIII^{glc}$, glucose-specific enzyme III of the PTS; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; decyl-PEG, decylpoly(ethylene glycol) 300; ISO, inside-out.

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Table I: Nucleotide and Amino Acid Replacements in *mtl*A Encoding Enzyme II^{mtl}

EII^{mtl} mutant	mutagenic primer[a]	new restriction site	amino acid replacement
H554A	5′-CAACCGTGCCGGCTGGCACC-3′	*Nae*I	His-554 → Ala
C384S	5′-CCGGCGTCGCTAGCAACGATG-3′	*Nhe*I	Cys-384 → Ser
D385I	5′-CCCATACCGGCGATACAGGCA-3′		Asp-385 → Ile

[a] Underlining indicates the nucleotides changed.

1990), confirm the location of P1 (His-554) on a fused EIII-like domain. In the following paper, we report the overexpression, purification, and characterization of the EIII-like domain of *E. coli* EII^{mtl} (van Weeghel et al., 1991). It can be produced as a stable protein which retains the ability to phosphorylate the second site at a considerable rate. The second phosphorylation site, P2, was located on a peptide covering the region Lys-379 to Met-393, which contained Cys-384, Asp-385, and Ser-390 and -391, all of which were potential sites for phosphorylation. The pH dependence of the hydrolysis of this phosphorylated peptide proved that the second phosphorylation site was an *S*-phosphocysteine (Pas & Robillard, 1988).

Whether phosphoryl group transfer between sites P1 and P2 occurs under physiological conditions within one subunit or via an intermolecular transfer in the native enzyme is unknown. Vogler et al. (1988) and Vogler and Lengeler (1988) reported that the phosphoryl group could be passed between different EII species, raising the possibility of a mechanistically significant intermolecular phosphoryl group transfer. These observations were supported in the case of *E. coli* EII^{mtl} when Stephan et al. (1989) and White and Jacobson (1990) demonstrated an intermolecular phosphotransfer between a truncated EII^{mtl}, missing the extreme C-terminal part, and an inactivated EII or large C-terminal fragment both containing the first phosphorylation site, His-554. In keeping with the results of Pas and Robillard (1988), a free Cys-384 was necessary for the activity to be observed.

This paper investigates the precise role of the phosphorylation sites in the phosphoryl group transfer within one subunit or between two subunits of the EII^{mtl} dimer, by producing site-directed EII mutants, and comparing the activities of these mutants with native EII^{mtl}.

Experimental Procedures

Materials

D-[1-^{14}C]Mannitol (59 mCi/mmol) and D-[1-^{3}H]mannitol (12 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. Restriction endonucleases, *E. coli* DNA polymerase (Klenow fragment), T_4 DNA ligase, and T_4 polynucleotide kinase were obtained from Boehringer Mannheim. Polyclonal antibodies against EII^{mtl} were raised in rabbits with purified EII^{mtl} as described by Pas et al. (1987). Goat anti-rabbit IgG–horseradish peroxidase conjugate was purchased from Bio-Rad. EI and HPr were purified as described previously (Dooyewaard et al., 1979). The sodium salt of mannitol 1-phosphate was prepared from the barium salt as described by Roossien et al. (1984). The mismatch oligonucleotides, obtained from Eurosequence BV Groningen, were prepared on an Applied Biosystems Model 380 B DNA synthesizer, completely deprotected and used unpurified. All other chemicals were reagent grade and were obtained from commercial sources.

Methods

Bacterial Strains. The following strains of *Escherichia coli* were used: JM101, Δ(*lac-pro*AB) *sup*E, *thi*, [F′, *tra*D36, *pro*A$^+$B$^+$, *lac*I^{q}ZΔM15] (Yanish-Perron et al., 1985); BMH71-18 MutL, Δ(*lac-pro*AB), *sup*E, *thi*, [F′, *pro*A$^+$B$^+$, *lac*I^{q}ZΔM15], *mut*L::Tn10 (Kramer et al., 1984); HB2154, Δ(*lac-pro*AB), *thi*, [F′, *pro*A$^+$B$^+$, *lac*I^{q}ZΔM15] (Carter et al., 1985); ASL-1, F$^-$, *lac*Y1, *gal*T6, *xyl*-7, *thi*-1, *his*G1, *arg*G6, *met*B1, *rps*L104, *mtl*A2, *rec*A (van Weeghel et al., 1990).

Oligonucleotide-Directed Mutagenesis. The mutagenic primers used to replace His-554, Cys-384, and Asp-385 in EII^{mtl} are listed in Table I. The gene encoding mannitol-specific EII (*mtl*A) has been subcloned and overexpressed in *E. coli* (van Weeghel et al., 1990). The whole gene with its own promoter was inserted into a phagemid mutagenesis vector, pMa5-8 (Stanssens et al., 1989). The resulting plasmid, pWAMa, produced high levels of phage particles in the medium, when the *E. coli* strain BMH 71-18MutL was superinfected with M13KO7 helper phage at a multiplicity of 20:1 when the bacterial cells were in the early log phase.

Isolation of single-stranded DNA (ssDNA) and oligonucleotide-directed mutagenesis were performed on ssDNA of pWAMa, following the procedure of the gapped duplex method with the pMac5-8 vector system, as described by Stanssens et al. (1989). The gapped duplex DNA (gdDNA) was made by mixing the linearized vector pMc5-8 with ssDNA of pWAMa (pMa5-8 type). Formation of gdDNA was checked by agarose gel electrophoresis of an aliquot of the DNA mixture. After annealing of the phosphorylated mismatch primer to the gdDNA, the gap is filled-in, in vitro, by DNA polymerase and T_4 DNA ligase. The resulting heteroduplex with the incorporated primer was transfected into the suppressor$^+$ (su$^+$) strain BMH71-18 MutL. An aliquot of the transformation mixture was plated on selective media to determine the efficiency of transformation. The remainder of the transformation mixture was used to inoculate 10 mL of 2×TY (16 g of Bacto tryptone, 10 g of yeast extract, and 5 g of NaCl per liter) medium containing the appropriate antibiotic. After overnight growth, plasmid DNA was isolated by using a small-scale purification method (Maniatis et al., 1982) and used to transform a suppressor$^-$ (su$^-$) strain such as HB2154. For selection of transformants, transformation mixtures were plated on 2×TY plates containing the appropriate antibiotic (chloramphenicol, 25 μg/mL). The entire *mtl*A gene encoding the H554A, C384S, or D385I mutation was sequenced by the dideoxynucleotide sequencing method (Sanger et al., 1977) using synthetic primers complementary to appropriate regions along the gene.

Growth of Cells and Preparation of Membranes. E. coli strain ASL-1, harboring the expression plasmids encoding wild-type EII^{mtl}, H554A, C384S, and D385I, respectively, was grown overnight in 2×TY medium (10 g of Bacto tryptone, 16 g of yeast extract, and 5 g of NaCl per liter) with the appropriate antibiotic. For preparation of inside-out (ISO) vesicles, cells were collected and disrupted by passage through a French pressure cell at 10000 psi. The suspension was centrifuged at 20000*g* for 10 min, to remove unbroken cells. The supernatant was recentrifuged at 200000*g* for 60 min, after which the supernatant was discarded and the pellet resuspended in buffer containing 50 mM Tris-HCl, pH 7.5, 1

mM DTT, and 1 mM EDTA (1 mL/g of starting cells, wet weight) and stored in aliquots in liquid nitrogen until used.

Mannitol Transport and Fermentation. EII^{mtl} permease activity was assayed qualitatively by transforming the EII^{mtl}-deficient strain ASL-1 with different expression plasmids, followed by growth and fermentation on mannitol-containing McConkey indicator plates (Difco Bacto Agar base, Difco Lab).

EII^{mtl} Activity Assays. PEP-dependent mannitol phosphorylation activity was measured as described by Robillard and Blaauw (1987). The assay mixture for the PEP-dependent phosphorylation contained 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM PEP, 5 mM DTT, and 0.35% decyl-PEG. The general phosphocarriers EI (0.6 μM) and HPr (3 μM) were present in saturating amounts, and ISO vesicles containing wild-type EII^{mtl}, EII-H554A, EII-C384S, and EII-D385I were added in different concentrations (1 nM up to 0.5 μM) to the assay mixture. The reaction mixtures were preincubated at 30 °C for 10 min; then reactions were started with 20 μM [^{14}C]mannitol. The buffer of the mannitol 1-phosphate/mannitol exchange assay was the same as for the PEP-dependent assay, except that 1 mM mannitol 1-phosphate was substituted for PEP, 0.5 μM [^{3}H]mannitol was used, and the general phosphocarriers, EI and HPr, were omitted.

In the complementation assays, PEP-dependent and exchange activities were measured under the same assay conditions as described above by adding different amounts of the EII-C384S mutant permease (3.4, 6.9, 17.3, 34.4, 68.8, and 137.5 nM) to the reaction mixture containing 5.6 nM EII-H554A mutant. The EII^{mtl} concentrations in the ISO vesicles were determined by measuring the specific mannitol binding using flow dialysis. Measurements were done at various mannitol concentrations, and the number of binding sites was determined by extrapolation from Scatchard plots. The EII concentration was calculated assuming one high-affinity binding site per dimer (Pas et al., 1988). Activities were calculated as a function of time at various enzyme concentrations and were linear with respect to both parameters.

Flow Dialysis. Binding of [^{3}H]mannitol to EII^{mtl} was measured with flow dialysis as described (Lolkema et al., 1990).

Protein Determination. Protein in the membrane vesicles was measured according to the method of Bradford (1976) with bovine serum albumin as a standard.

Results

Mutagenesis, DNA Sequencing, and Expression. The *mtl*A gene encoding the mannitol-specific EII was subcloned onto a phagemid vector to yield the plasmid pWAMa which was used, after transforming it into *E. coli* JM101, to isolate dsDNA and ssDNA needed for gapped-duplex mutagenesis with amber selection (Figure 1). Together with the replacements of specific amino acids, new restriction sites were introduced into the gene for easy identification of the mutated plasmids. After the mutagenesis reactions were performed, plasmid DNA was isolated from randomly picked colonies and used for restriction analysis for the presence of the new sites and corresponding amino acid substitutions (Table I). A few clones of each mutant containing mutant plasmid DNA were infected with helper phage for the production of ssDNA. Subsequently, phages containing mutated *mtl*A were isolated, and their ssDNA was sequenced with appropriate primers (Sanger et al., 1977). The amino acid substitutions, corresponding restriction sites, and primers are listed in Table I. In codon 554, the histidine was replaced by Ala, while in codons 384 and 385 the Cys and Asp were replaced by Ser and Ile, respectively. The entire *mtl*A gene of all mutants was also sequenced; with the exception of the desired substitutions, the gene sequence was identical with that reported by Lee and Saier (1983).

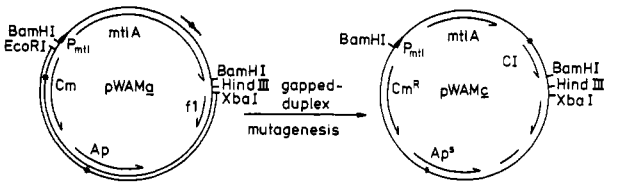

FIGURE 1: Oligonucleotide-directed mutagenesis following the gapped-duplex method with amber selection using the pMac5-8 system. The phagemid pWAMa, containing the gene *mtl*A encoding wild-type EII^{mtl}, was used as template to generate different mutations with concomitant amino acid substitutions. After annealing of the mismatch primer and filling-in the gap in vitro, the heteroduplex molecule was transformed into an *E. coli* strain for selection of the DNA strand containing the mismatch. The asterisk stands for an amber mutation in the resistance genes.

In vivo mannitol transport and fermentation catalyzed by mutated EII's were monitored in *E. coli* ASL-1 (*mtl*A$^-$). The strain was transformed with the expression plasmids encoding wild-type EII^{mtl} or the mutated enzyme, EII-H554A,[2] EII-C384S, and EII-D385I, and analyzed qualitatively on McConkey indicator plates containing the appropriate antibiotic and 0.2% D-mannitol. After growth overnight, transformants harboring wild-type EII^{mtl} were dark red, indicating the uptake and fermentation of the sugar. As expected, transformants of the two phosphorylation site mutants, EII-H554A (P1) and EII-C384S (P2), were white, indicating that these enzymes were not able to transport and phosphorylate mannitol needed for fermentation. Contrary to our expectations, EII-D385I transformants were also white on the indicator plates, suggesting a negative mannitol fermentation phenotype. Although, no fermentation on indicator plates was detectable for these transformants, differences became apparent when the membranes were isolated and assayed for mannitol phosphorylation in vitro.

In order to compare the specific activities of the mutants and wild-type EII^{mtl} in vitro, it was essential to quantitate the EII^{mtl} content in the membranes. EII concentration was established by analyzing small aliquots of ISO vesicles by flow dialysis. Data obtained by this procedure showed some differences in the expression levels of the various mutated forms of EII^{mtl}, even though they were expressed from the same promoter (Figure 1). The amount of wild-type EII, EII-H554A, and EII-C384S in the ISO vesicle preparations was 1.7, 2.8, and 0.7 μM, respectively. The concentration of EII-D385I could not be accurately determined by using this mannitol binding method due to nonlinear Scatchard plots.

PEP-Dependent Phosphorylation and Transphosphorylation of Mannitol in Vitro. Membrane preparations from ASL-1 harboring different EII^{mtl} mutants were tested for their ability to catalyze PEP-dependent phosphorylation, via P1 and P2 (Scheme I and Figure 2), or to catalyze mannitol/mannitol phosphate exchange via P2 (Scheme II). Table II summarizes the results of the experiments. The two phosphorylation site mutants, EII-H554A and EII-C384S, which showed no mannitol fermentation on McConkey plates, showed only background PEP-dependent phosphorylation activity in vitro. These results were expected since these sites

[2] Site-directed mutants are designated as follows: The one-letter amino acid code is used followed by a number indicating the position of the residue in the wild-type permease, then followed by a second letter denoting the amino acid replacement at this position.

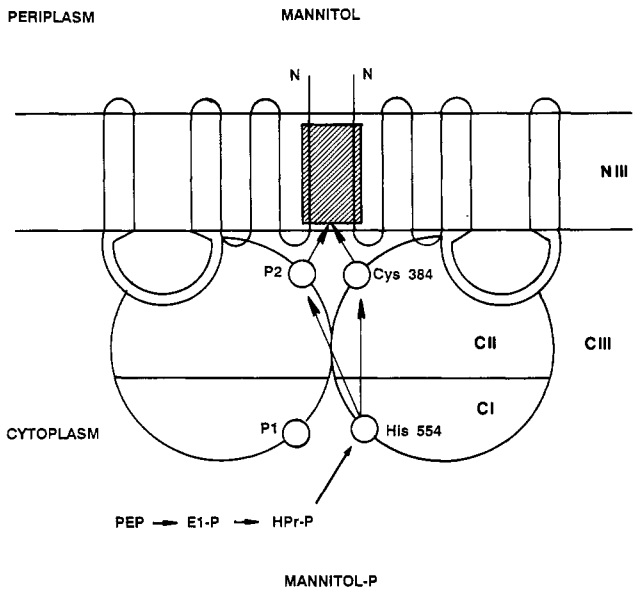

FIGURE 2: Schematic representation of an EII^{mtl} dimer and possible routes of the phosphoryl group from phosphoenolpyruvate to mannitol, indicated by the direction of the arrows. NIII represents the membrane-bound domain containing the sugar binding site. CI and CII represent two separate domains within the cytoplasmic domain, containing the two active-site residues His-554 (P1) and Cys-384 (P2). In the EII-H554A mutant, the His-554 is replaced by alanine, while in the EII-C384S mutant the Cys-384 is replaced by a serine.

Table II: PEP-Dependent Phosphorylation and Exchange Activities of Different Enzyme II Mutants in Vitro

EII^{mtl} with amino acid replacement[a]	plasmid[b]	sp act.[c] [nmol of mtl-P min^{-1} (nmol of EII^{mtl})$^{-1}$] PEP-dependent phosphorylation	exchange
wild type	pWAMa	855	9.7
H554A	pWAMc7	0.12	12.0
c384S	pWAMc5	0.20	0.048
D385I	pWAMc1	++[d]	++[d]

[a] See Table I. [b] EII^{mtl} mutants are all expressed from the same vector system as depicted in Figure 1. [c] The conditions and protein concentrations used are specified under Experimental Procedures. [d] The concentration of EII-D385I could not be accurately determined by using the mannitol binding method due to nonlinear Scatchard plots. Consequently, quantitative statements cannot be made.

have been identified chemically as the phosphorylation sites.

The Cys-384 peptide has an aspartic acid at position 385 which, in principle, could also function as a phosphorylation site, as is the case in Na^+/K^+-ATPase (Post & Kume, 1973) and the CheY protein (Sanders et al., 1989). Considering the novelty of the phosphocysteine observation (Pas & Robillard, 1988a), replacement of the Asp seemed like a prudent control experiment. The choice of Ile as a replacement for Asp-385 was dictated by the occurrence of the sequence Ala-Cys-Ile in EII^{bgl}, EII^{nag}, and EII^{man} in stretches homologous to the phosphorylation site peptide containing an Ala-Cys-Asp sequence in EII^{mtl} and a similar peptide in EII^{gut}. Table II shows that the activity of EII-D385I could be measured and, therefore, Asp-385 does not appear to be essential for catalytic activity.

The presence of the second phosphorylation site, Cys-384, is a prerequisite for exchange between mannitol and mannitol 1-phosphate (Scheme II and Figure 2). The exchange activity of the wild-type EII^{mtl}, under the conditions specified under Experimental Procedures, was 9.7 nmol min^{-1} (nmol of EII^{mtl})$^{-1}$, while EII-C384S, as expected, was completely inactive in this in vitro assay (Table II). Under the same conditions, the rate of exchange of the EII-H554A permease was increased up to 12.0 nmol min^{-1} nmol^{-1}. Membranes containing EII-D385I were also active in the exchange reaction.

Table III: PEP-Dependent Phosphorylation Activities of Different Enzyme II Mutants in Vitro by Complementation

[EII-H554A] (nM)	[EII-C384S] (nM)	sp act.[a] PEP-dependent phosphorylation [nmol of mtl-P min^{-1} (nmol of EII-H554A)$^{-1}$]
5.6		0.0
	34.4	0.0
5.6	3.4	57.3
5.6	17.3	137.2
5.6	34.4	212.1
5.6	68.8	281.7
5.6	137.5	371.2

[a] The activities are corrected for background, 0.12 nmol of mtl-P min^{-1} (nmol of EII-H554A)$^{-1}$ and 0.2 nmol of mtl-P min^{-1} (nmol of EII-C384S)$^{-1}$.

Complementation of PEP-Dependent Phosphorylation Activity. Figure 2 indicates two possible routes of phosphoryl group transfer between sites P1 and P2: a direct transfer between His-554 (P1) and Cys-384 (P2) within one subunit and a transfer between these two sites on two different subunits. We have examined the ability of EII-C384S to catalyze the phosphorylation of the EII-H554A active-site cysteine via a possible intermolecular phosphotransfer between two subunits. The two mutant proteins are separately incapable of mannitol phosphorylation (see Table III). In order to determine the efficacy of EII-C384S in restoring the activity of the EII-H554A mutant, its concentration dependence in mannitol phosphorylation was measured. ISO vesicles containing EII-H554A (5.6 nM) were combined with various amounts of ISO vesicles containing EII-C384S (3.4, 6.9, 17.3, 34.4, 68.8, and 137.5 nM), solubilized in decyl-PEG, and tested for PEP-dependent phosphorylation activity in the presence of saturating amounts of PEP, EI, and HPr. The data listed in Table III show that increasing concentrations of EII-C384S resulted in an increase of activity. The data were plotted in a double-reciprocal plot, yielding a K_m for EII-C384S on EII-H554A of 42 nM and a V_{max} of 475 nmol min^{-1} (nmol of EII-H554A)$^{-1}$, which is half of the wild-type rate of 855 nmol min^{-1} (nmol of EII)$^{-1}$ assayed under the same conditions.

Complementation of Mtl/Mtl-P Exchange Activity. The data of the in vitro exchange experiments shown in Table II clearly demonstrate that the mutant EII-C384S is completely inactive but that the EII-H554A has retained its ability to catalyze the mannitol/mannitol phosphate exchange. Even though this enzyme retains a high level of exchange activity, the activity can be increased further by addition of the inactive EII-C384S. This is shown by the data in column 6 of Table IV. In contrast, addition of ASL-1 ISO vesicles lacking EII^{mtl} does not stimulate the exchange activity of EII-H554A, but even lowers it, suggesting that the increase in exchange activity is specific for the presence of EII-C384S. Since EII-C384S by itself is inactive in the exchange reaction, we conclude that the increased activity is due to the formation of heterodimers.

Discussion

Dimeric forms of EII^{mtl} have been observed and reported to be important for the structure and function of the enzyme (Leonard & Saier, 1983; Roossien & Robillard, 1984; Stephan & Jacobson, 1986; Pas et al., 1988; Lolkema & Robillard, 1990). Figure 2 presents a schematic representation of the dimer protein and shows the residues identified as the two phosphorylated intermediates. His-554, which is located in

Table IV: Exchange Activities of Different Enzyme II Mutants in Vitro by Complementation

	EII dimer concn[a]						
ASL[c]	H554A (nM)	C384S (nM)	H554A [homodimer]	C384S [homodimer]	H554A/C384S [heterodimer]	exchange act.[b] (pmol/min)	sp exchange act.[b] [nmol min^{-1} (nmol of homo+heterodimer containing EII-H554A)$^{-1}$]
	5.6		5.6			6.7	12.0
		34.4		34.4		0.14	0.00
	5.6	3.4	3.48	1.28	4.23	7.2	9.34
	5.6	6.9	2.51	3.81	6.18	7.8	8.98
	5.6	17.3	1.39	13.06	8.46	9.3	9.46
	5.6	34.4	0.78	29.58	9.63	10.8	10.37
++	5.6		5.6			4	7.1

[a] These are theoretical values calculated by assuming identical equilibrium constants for the formation of homo- and heterodimers and assuming that all enzyme is in the dimer form. The concentration of EII^{mtl} in the different ISO vesicles was determined by flow dialysis experiments as described under Experimental Procedures. [b] Buffer conditions and protein concentrations used in the complementation assays are described under Experimental Procedures. [c] The concentration of total protein in the experiments using ASL ISO vesicles lacking EII^{mtl} was identical with the concentration of total protein in the experiments using ISO vesicles containing 34.4 nM EII-C384S.

an EIII-like domain (Pas et al., 1988; Grisafi et al., 1989; van Weeghel et al., 1991), receives its phosphoryl group from phospho-HPr and passes it on to the second phosphorylation site, Cys-384. From there, the phosphoryl group is coupled to mannitol. This is the normal mannitol transport and phosphorylation sequence followed in vivo (Scheme I). The enzyme is also capable of catalyzing the exchange reaction (Scheme II) in the absence of other PTS components. For this reaction, it is assumed that mannitol 1-phosphate binds to the normal product-leaving site and phosphorylates Cys-384, after which the same or another molecule of mannitol dephosphorylates the enzyme forming mannitol-1-P again. Conceptually, the active-site residue, His-554, is not necessary for this reaction.

The results presented here support the conclusions of Pas and Robillard (1988) that Cys-384 is important for phosphorylation of mannitol. These results are in agreement with data reported by Nuoffer et al. (1988), where they inactivated the glucose permease in the PEP-dependent phosphorylation reaction upon replacing the activity-linked cysteine with Ser, and with results presented by Grisafi et al. (1989), where they produced and characterized different classes of EII^{mtl} C-terminal deletion mutants. The only class of truncated mannitol permeases which retain phosphorylation activity were those with the Cys-384 and His-554 preserved. The His to Ala substitution in EII-H554A led to the complete inactivation of the in vivo and in vitro PEP-dependent phosphorylation activity, because phosphoryl group transfer from phospho-HPr to EII^{mtl} is unable to occur. In principle, the mannitol/mannitol phosphate exchange activity of EII-H554A would not have to be reduced if the His-554 phosphorylation site were not necessary for this reaction. The exchange activity listed in Table II confirms this proposal. The increase in specific activity to 12.0 nmol min^{-1} nmol^{-1}, compared to the 9.7 nmol min^{-1} nmol^{-1} wild-type EII^{mtl}, indicates that the domain carrying His-554 interacts with and influences the activity of the domain carrying the Cys-384. Grisafi et al. (1989) observed increased activities in more than half of the deletion mutants in which segments extending from the C-terminus (637) into residue 520 had been deleted; however, since the EII concentration of their mutants was not determined, quantitative statements could not be made.

Complementation of the PEP-Dependent Phosphorylation Activity of the EII-H554A and EII-C384S Permeases. The present data clearly demonstrate an intermolecular phosphotransfer between subunits of the two permeases. This result is consistent with observations made by Stephan et al. (1989) and White and Jacobson (1990), who showed phosphotransfer between a truncated EII^{mtl}, missing the first phosphorylation site, and a proteolytic and partially purified C-terminal fragment or an expressed cytoplasmic domain of EII^{mtl}. It is also consistent with the results of Vogler et al. (1988) and Vogler and Lengeler (1988). They reported that EII^{nag} and EII^{bgl}, whose C-terminal domains show considerable homology with EIII^{glc}, could replace the EIII^{glc} in EII^{glc}-dependent glucose transport and phosphorylation. Furthermore, addition of EIII^{glc} to a truncated EII^{nag} restored the activity of the mutated EII^{nag}.

The mannitol phosphorylation rates using P1 and P2 site mutants show that intermolecular phosphotransfer occurs at a considerable rate. The calculated V_{max} of 475 nmol min^{-1} (nmol of EII-H554A)$^{-1}$ is half of that of the wild-type [855 nmol min^{-1} (nmol of EII)$^{-1}$]. In the following paper (van Weeghel et al., 1991), we demonstrate the phosphotransfer between a purified domain (CI) containing the first phosphorylation site, His-554 (Figure 2), and EII-H554A described in this paper. The K_m and V_{max} were 26.4 μM and 212 nmol min^{-1} (nmol of EII-H554A)$^{-1}$, respectively, which lead to the conclusion that there is a distinct binding site for the CI domain on the CII domain involved in transferring the phosphoryl group to Cys-384. The results of the domain study and the present study with intact EII mutants differ in that the PEP-dependent phosphorylation activity of EII-H554A could be restored by much lower concentrations of EII-C384S mutant (K_m = 42 nM) than of purified CI domain (K_m = 26.4 μM). Furthermore, the V_{max} was twice as high with the intact mutants. Although this is not proof of the formation of stable heterodimers, it shows that the phosphotransfer between intact enzymes is much better, probably due to a stronger and more selective association between the intact proteins.

Complementation of the Mtl/Mtl-P Exchange Activity of EII-H554A and EII-C384S. EII^{mtl} concentration-dependent kinetics have suggested that subunit association (dimers or oligomers) is essential for exchange activity (Leonard & Saier, 1983; Roossien et al., 1984; Lolkema & Robillard, 1990). If we assume that exchange occurs only with stable EII dimers, the activity in Table IV would be due to EII-H554A homodimers and EII-H554A/EII-C384S heterodimers. When the exchange activity is expressed in terms of the concentration of these dimers, we see an increase by a factor of 1.3–1.5. The simplest explanation for the necessity of dimers for the exchange activity is that one subunit binds mannitol phosphate while the other binds mannitol. Since phosphoryl transfer occurs via Cys-384, both of the subunits but only one of the

cysteines would be involved in each turnover of the homodimer. In the heterodimers, only one Cys-384 is present per dimer, thus allowing every cysteine to participate in every turnover. This would result in twice as much mannitol phosphate being formed per unit time. The maximum stimulation expected would be a factor of 2. That the maximum stimulation is not achieved is understandable since nonproductive complexes in which mannitol phosphate binds to the "incorrect" subunit are expected.

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REFERENCES

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Carter, P., Bedouelle, H., & Winter, G. (1985) *Nucleic Acids Res. 13*, 4431–4444.
Dooyewaard, G., Roossien, F. F., & Robillard, G. T. (1979) *Biochemistry 18*, 2990–2996.
Fischer, R., Eisermann, R., Reiche, B., & Hengstenberg, W. (1989) *Gene 82*, 249–257.
Grisafi, P. L., Scholle, A., Sugayama, J., Briggs, L., Jacobson, G. R., & Lengeler, J. W. (1989) *J. Bacteriol. 171*, 2719–2727.
Jacobson, G. R., Lee, C. A., & Saier, M. H., Jr. (1979) *J. Biol. Chem. 254*, 249–252.
Kramer, B., Kramer, W., & Fritz, H. J. (1984) *Cell 38*, 879–887.
Lee, C. A., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10761–10767.
Leonard, J. E., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10757–10760.
Lolkema, J. S., & Robillard, G. T. (1990) *Biochemistry 29*, 10120–10126.
Lolkema, J. S., Swaving Dijkstra, D., Ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1990) *Biochemistry 29*, 10659–10663.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Nuoffer, C., Zanolari, B., & Erni, B. (1988) *J. Biol. Chem. 263*, 6647–6655.
Pas, H. H., & Robillard, G. T. (1988) *Biochemistry 27*, 5835–5839.
Pas, H. H., Ellory, J. C., & Robillard, G. T. (1987) *Biochemistry 26*, 6689–6696.
Pas, H. H., Ten Hoeve-Duurkens, R. H., & Robillard, G. T. (1988) *Biochemistry 27*, 5520–5525.
Post, R. L., & Kume, S. (1973) *J. Biol. Chem. 248*, 6993–7000.
Postma, P. W., & Lengeler, J. W. (1985) *Microbiol. Rev. 49*, 232–269.
Reiche, B., Frank, R., Deutscher, J., Meyer, N., & Hengstenberg, W. (1988) *Biochemistry 27*, 6512–6516.
Robillard, G. T., & Blaauw, M. (1987) *Biochemistry 26*, 5796–5803.
Robillard, G. T., & Lolkema, J. S. (1988) *Biochim. Biophys. Acta 947*, 493–519.
Roossien, F. F., & Robillard, G. T. (1984) *Biochemistry 23*, 5682–5685.
Roossien, F. F., Blaauw, M., & Robillard, G. T. (1984) *Biochemistry 23*, 4934–4939.
Sanders, D. A., Gillece-Castro, B. L., Stock, A. M., Burlingame, A. L., & Koshland, D. E. (1989) *J. Biol. Chem. 264*, 21770.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., & Fritz, H. J. (1989) *Nucleic Acids Res. 17*, 4441–4454.
Stephan, M. M., & Jacobson, G. R. (1986) *Biochemistry 25*, 4046–4051.
Stephan, M. M., Khandekar, S. S., & Jacobson, G. R. (1989) *Biochemistry 28*, 7941–7946.
van Weeghel, R. P., Keck, W., & Robillard, G. T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2613–2617.
van Weeghel, R. P., Meyer, G. H., Keck, W., & Robillard, G. T. (1991) *Biochemistry* (following paper in this issue).
Vogler, A. P., & Lengeler, J. W. (1988) *Mol. Gen. Genet. 213*, 175–178.
Vogler, A. P., Broekhuizen, C. P., Schuitema, A., Lengeler, J. W., & Postma, P. W. (1988) *Mol. Microbiol. 2*, 719–726.
White, D. W., & Jacobson, G. R. (1990) *J. Bacteriol. 172*, 1509–1515.
Yanish-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103–119.